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Immunology

Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane

(alternative complement pathway/β1H/C3b inactivator/properdin)

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ABSTRACT **An activity that is inhibitory to the properdin-stabilized amplification C3 convertase (C3b,Bb,P) was solubilized from human erythrocyte (E^{hu}) membranes by Nonidet P-40 and purified to homogeneity. The inhibitory membrane glycoprotein had an apparent M_r of 1–1.2 × 10^6 on gel filtration in the presence of Nonidet P-40. On sodium dodecyl sulfate/polyacrylamide gel electrophoresis it presented a single stained band with an apparent M_r of 205,000, with or without prior reduction of disulfides. The inhibitory protein of the E^{hu} membrane produced a dose-related, first-order decay of C3b,Bb,P function on sheep erythrocytes (E^s) and released ^{125}I-labeled Bb from these sites, indicating a mechanism of inhibition by decay-dissociation of the amplification C3 convertase. The 50% inhibitory dose of the E^{hu} membrane protein was not altered by removal of sialic acid from the E^s bearing C3b,Bb,P sites. E^{hu} membrane protein also serves as a cofactor for C3b inactivator-induced cleavage of the α polypeptide chain of C3b. Thus, the inhibitory membrane protein can abrogate the activity of amplification convertase sites that have formed and also can prevent generation of such sites by augmenting irreversible inactivation of C3b.**

Discrimination between cells by the alternative complement pathway occurs after initial deposition of C3b and is related to the modulation by surface constituents of the capacity of bound C3b to function as a subunit of the amplification C3 convertase. The existence in the E^{hu} membrane of a protein that can impair the functions of membrane-bound C3b and C3b,Bb,P could represent a molecular basis for preventing inappropriate self-recognition.

The alternative pathway of human complement, which is composed of six plasma proteins, C3, B, $\overline{D}$, properdin (P), C3b inactivator (C3bINA), and β1H, can discriminate between one cell and another in the absence of specific antibody. Recognition of a cell by this pathway eventuates in deposition on the membrane of C3b, the major cleavage fragment of C3, and the C5b-9 molecular complex with attendant implications for phagocytosis and cytolysis, respectively. The slow and continuous interaction of C3, B, $\overline{D}$, and P generates small amounts of C3b (1, 2), which may covalently attach (3) to bystander surfaces. The cell-bound C3b then complexes with B; cleavage-activation of the latter by $\overline{D}$ generates the amplification convertase, C3b,Bb (4, 5), which can be stabilized by uptake of P (6, 7). The C3b,Bb sites cleave C3 and deposit additional C3b, thereby accelerating the reaction. This positive feedback mechanism is regulated by binding of the control protein, β1H (8–11), to C3b, which prevents uptake of B at that site (11), facilitates decay-dissociation of Bb that is already complexed to C3b (12), and enhances proteolytic inactivation of C3b by C3bINA (8, 13).

Whether a cell is recognized by the alternative complement pathway is determined by the capacity of its surface to regulate formation and function of bound C3b,Bb sites (14, 15). Membrane-associated sialic acid promotes high-affinity binding of β1H to C3b without influencing the affinity of B for C3b (11), accounting for the observed inverse relationship between membrane content of sialic acid of sheep (16) and mouse erythrocytes (17) and the lysis of these cells in whole human serum by activation of the alternative complement pathway. In an analogous manner, heparin glycosaminoglycan that is coupled to the surface of zymosan particles suppresses recognition by the human alternative pathway through a dose-dependent facilitation of the regulatory action of the β1H and C3bINA on particle-bound C3b (18). Thus, sialic acid and an *N*-sulfated mucopolysaccharide are two surface constituents that impair formation and function of bound C3b,Bb sites by an indirect mechanism involving the fluid-phase control proteins.

Although relative deficiencies of cell-surface sialic acid and sulfated mucopolysaccharides are biochemical characteristics that can be recognized by the alternative complement pathway to promote the phagocytosis and cytolysis of foreign cells in the nonimmune host, the failure of human serum to lyse normal human erythrocytes (E^{hu}) that have been desialated indicates the existence of additional surface-associated entities with regulatory functions. A protein of E^{hu} membranes has been purified to homogeneity and shown to impair directly the formation and function of the human C3b,Bb. This membrane protein accelerates decay-dissociation of C3b,Bb sites and serves as a cofactor for C3bINA-dependent cleavage of C3b.

MATERIALS AND METHODS

Alternative Complement Pathway Proteins and Hemolytic Assays. Alternative complement pathway proteins B (19), $\overline{D}$ (6), C3 (20), P (14), β1H (12), and C3bINA (21) were purified to homogeneity, as assessed by polyacrylamide gel electrophoresis of their disulfide-reduced forms in the presence of sodium dodecyl sulfate ($NaDodSO_4$). C3 and B were trace-labeled with ^{125}I (New England Nuclear) by use of insolubilized lactoperoxidase (22) (Worthington) to specific activities of 84,000 cpm/μg and 72,000 cpm/μg, respectively, without loss of function. Two hundred micrograms of ^{125}I-labeled C3 was converted to ^{125}I-labeled C3b by incubation for 60 min at 30°C with 200 μg of B and 1 μg of $\overline{D}$ in 0.5 ml of Veronal-buffered saline (pH 7.5) (VBS) containing 1 mM $MgCl_2$ (1). ^{125}I-Labeled C3b was isolated by gel filtration of the reaction mixture on Sephadex G-200 (Pharmacia) equilibrated in VBS. VBS con-

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Abbreviations: C3b,Bb, amplification C3 convertase formed from the major cleavage fragments of C3 and B; C3b,Bb,P, properdin-stabilized C3b,Bb; C3bINA, C3b inactivator; E^{hu}, human erythrocytes; E^s, sheep erythrocytes; $E^sAC4b,3b$, E^s bearing rabbit antibody and the major cleavage fragments of C4 and C3; NP-40, Nonidet P-40; P, properdin; $NaDodSO_4$, sodium dodecyl sulfate; VBS, Veronal-buffered saline; GVB, VBS containing 0.1% gelatin; $DGVB^{++}$, half-isotonic GVB containing 2.5% dextrose, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$.

taining 0.1% gelatin (GVB) and half-isotonic GVB containing 2.5% dextrose, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$ ($DGVB^{++}$) were used as diluents (23) in hemolytic assays.

Solubilized proteins of E^{hu} membranes or chromatographic fractions of these proteins were assessed for their capacity to decay-dissociate P-stabilized amplification convertase sites (C3b,Bb,P) formed on sheep erythrocytes (E^s) (12). E^s bearing rabbit antibody and the major cleavage fragments of C4 and C3 ($E^sAC4b,3b$), 5×10^7 cells per ml of $DGVB^{++}$, were incubated for 30 min at 30°C with 1 μg of P per ml, 0.05 μg of $\overline{D}$ per ml, and sufficient B to generate approximately two effective C3b,Bb,P sites per cell. $E^sAC4b,3b,Bb,P$ (1×10^7) in 0.2 ml of $DGVB^{++}$ were incubated with dilutions of solubilized E^{hu} membrane proteins in 0.1 ml of GVB containing 20 mM EDTA or with buffer alone for 20 min at 30°C. Residual C3b,Bb sites were then developed by addition of 0.3 ml of a 1:20 dilution of rat serum in GVB containing 40 mM EDTA and incubation at 37°C for 60 min. The percent hemolysis of the cellular intermediates was determined and the average number of hemolytic sites per cell was calculated.

C3b,Bb sites were also formed on E^s and E^s lacking 71% of membrane sialic acid. E^s (1×10^{10}) were incubated for 30 min at 37°C with 0.1 unit of purified *Clostridium perfringens* sialidase (16) (acylneuraminyl hydrolase, EC 3.2.1.18) in 1 ml of 10 mM sodium phosphate/150 mM NaCl, pH 6.5. The desialated E^s were sedimented by centrifugation at $1300 \times g$ for 10 min, and the supernatant was removed for assay of sialic acid by the thiobarbituric acid method (24) and compared with a standard curve established with synthetic *N*-acetylneuraminic acid (Calbiochem). E^s and desialated E^s were converted to C3b-bearing intermediates by a two-step procedure (16). Samples of each cell type (5×10^9 cells) were incubated with 1000 μg of B, 1200 μg of C3, and 1 μg of $\overline{D}$ in 1 ml of $DGVB^{++}$ for 45 min at 30°C and washed four times with GVB. The cells from this first step were incubated for 30 min at 30°C with 50 μg of B and 0.5 μg of $\overline{D}$ in 1 ml of $DGVB^{++}$, pelleted by centrifugation at 2°C, resuspended in 1 ml of $DGVB^{++}$ containing 500 μg of C3, and incubated an additional 45 min at 30°C. After being washed four times with GVB, E^sC3b and desialated E^sC3b were converted to cellular intermediates bearing C3b,Bb,P sites by the same procedure that has been described for $E^sAC4b,3b$.

Preparation of E^{hu} Membranes and Affinity Chromatographic Material. E^{hu} (10^{13}) were lysed by addition to 10 liters of 5 mM sodium phosphate/0.5 mM EDTA, pH 7.8, at 2°C, and the membranes were collected by centrifugation at $48,000 \times g$ for 30 min at 2°C. The membranes were washed four times until free of hemoglobin by resuspension in 10 liters of this buffer containing 0.5 mM phenylmethylsulfonyl fluoride and centrifugation. The protein content of the membranes and chromatographic fractions of solubilized proteins was estimated by the Lowry assay (25), with reference to a standard curve established with human serum albumin. Insoluble material that appeared during determination of the protein content of solutions containing Nonidet P-40 (NP-40, Gallard–Schlesinger, Carle Place, NY) was removed by centrifugation prior to spectrophotometric measurement. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed (26) with samples of proteins containing 2% $NaDodSO_4$ and 4 M urea, with and without addition of 0.2 M 2-mercaptoethanol, that had been incubated for 3 min in a boiling water bath. Standards for M_r estimation by $NaDodSO_4$/polyacrylamide gel electrophoresis were reduced and unreduced C3, β1H, and B and human serum albumin and its dimer, trimer, and tetramer. Proteins were detected in the gels by staining with Coomassie blue.

Sepharose-C3 was prepared by coupling 20 mg of C3 to 10 ml of CNBr-activated (27) Sepharose-4B (Pharmacia). Sepharose-lentil lectin was prepared by coupling 20 mg of the lectin (Sigma) to 10 ml of CNBr-activated Sepharose 4B in the presence of 0.1 M α-methylmannoside.

RESULTS

Purification of Inhibitory Protein of E^{hu}. Membranes from 1×10^{13} E^{hu}, which had a total protein content of 4820 mg, were suspended in 1 liter of 5 mM sodium phosphate/0.5 mM EDTA/150 mM NaCl/1% NP-40, at pH 7.5, and stirred overnight at 4°C. The solution was freed of insoluble material by centrifugation at $48,200 \times g$ for 30 min at 2°C. The supernatant, containing 2034 mg, was diluted with sufficient ice-cold H_2O to lower the conductivity to 6 mS at 0°C and was applied to a 2.6×30 cm column containing Biorex-70, 200–400 mesh (Bio-Rad) that had been equilibrated with 50 mM sodium phosphate/20 mM NaCl/0.2% NP-40, at pH 7.2. The column was washed with 200 ml of the equilibrating buffer and was eluted by application of a linear NaCl gradient of 500 ml, with the limit buffer containing 600 mM NaCl. A single peak of inhibitory activity for the C3b,Bb,P on E^s eluted between 8 and 11 mS. A pool of 60 ml, containing 30% of the inhibitory activity and 2% of the applied protein, was concentrated to 20 ml by positive pressure ultrafiltration by use of an Amicon PM-30 membrane (Amicon, Lexington, MA). After addition of 100 ml of ice-cold H_2O, the solution was applied at 4°C to a 1.6×4 cm column containing Sepharose-C3 that had been equilibrated with 10 mM sodium phosphate (pH 7.5). The column was washed sequentially with 50 ml of the equilibrating buffer, with buffer containing 0.1% NP-40, with buffer containing 200 mM NaCl, and with buffer containing both 0.1% NP-40 and 200 mM NaCl. Eighty-two percent of the inhibitory activity and 9% of the applied protein eluted from the column with the final

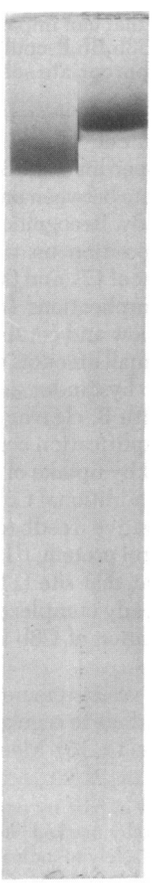

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of the purified inhibitory protein of the E^{hu} membrane in its unreduced (*Left*) and disulfide-reduced (*Right*) forms.

buffer. This pool was concentrated to 3 ml by positive pressure ultrafiltration and applied to a 2.6 × 90-cm column containing Bio-Gel A-5m (Bio-Rad) that had been equilibrated in VBS containing 0.1% NP-40. Inhibitory activity filtered at 65% of the bed volume, coincident with a distinct protein peak and corresponding to an apparent M_r of 1–1.2 × 10^6. The pool that contained 90% of inhibitory activity and 13% of applied protein was subjected to affinity chromatography, with 0.8 ml of Sepharose-lentil lectin, in a pasteur pipette. The column was washed with 10 ml of 10 mM Tris·HCl/200 mM NaCl/0.1% NP-40/0.7 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$ at pH 7.4, and eluted with this buffer containing 200 mM α-methylmannoside. Inhibitory activity appeared only in the eluate; fractions comprising 51% and 30% of applied activity and protein, respectively, were combined and dialyzed against VBS containing 0.1% NP-40. This final pool contained 150 μg of protein and 11.5% of the inhibitory material initially solubilized from the E^{hu} membranes, representing a 1575-fold purification.

Two samples of 15 μg of purified inhibitory protein of E^{hu} membranes, with and without prior reduction with 0.2 M 2-mercaptoethanol, were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis on 4.5% gels. Single stained bands of similar mobilities were apparent in both gels, suggesting that the inhibitory membrane protein consists of a single polypeptide chain (Fig. 1). By comparison with the mobilities of proteins with known M_r, the reduced form of the inhibitory protein had an apparent M_r of 205,000.

Decay-Dissociation of C3b,Bb by the Inhibitory Protein of E^{hu} Membrane. The dose–response effects of the purified inhibitory protein of the E^{hu} membrane were studied in a kinetic experiment. E^sAC4b,3b (2.5 × 10^8) were incubated with 5 μg of P, 0.25 μg of $\overline{D}$, and 25 ng of B in 5 ml of $DGVB^{++}$ for 30 min at 30°C to generate E^sAC4b,3b,Bb,P. The cellular intermediates were washed once in ice-cold $DGVB^{++}$, and replicate samples of 6 × 10^7 cells were resuspended in 1.2 ml of GVB with 20 mM EDTA alone or containing 24, 8, or 2.7 ng of the inhibitory membrane protein per ml. Incubation was

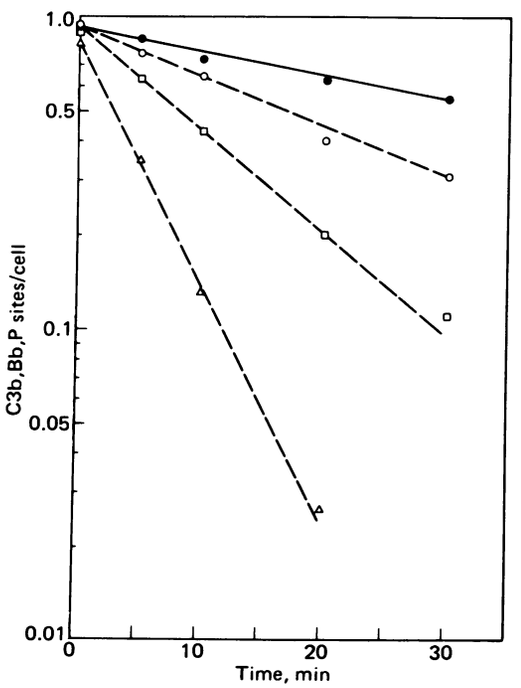

FIG. 2. Decay of C3b,Bb,P sites on E^s alone (●—●) and in the presence of 2.7 (○---○), 8 (□---□), and 24 (△---△) ng of the inhibitory protein of the E^{hu} membrane per ml.

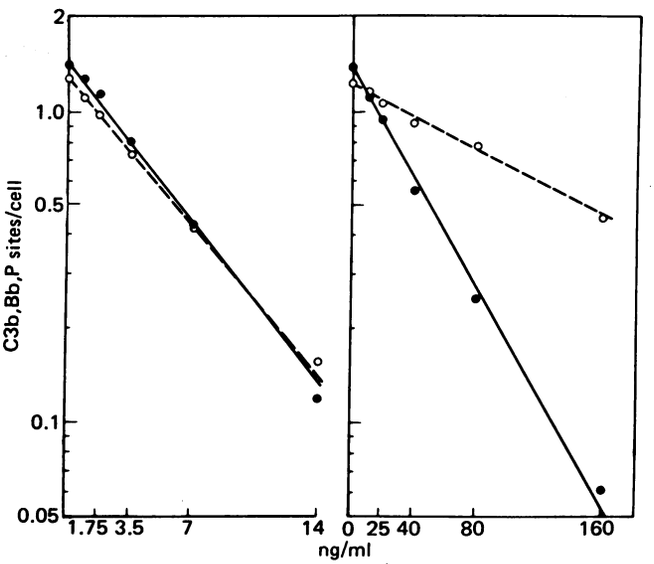

FIG. 3. Residual C3b,Bb,P sites per cell on E^s (●—●) and desialated E^s (○---○) after incubation with increasing concentrations of the inhibitory protein of the E^{hu} membrane (*Left*) and β1H (*Right*).

continued at 30°C, during which 0.2-ml samples were removed from each reaction mixture at timed intervals and added to 0.3 ml of rat serum diluted 1:20 in GVB with 40 mM EDTA to develop hemolytically the residual C3b,Bb,P sites during incubation at 37°C for 60 min. The half-life of the C3b,Bb,P sites on E^s in buffer alone was 42 min and decreased in a dose-related manner with increasing concentrations of the inhibitory membrane protein (Fig. 2).

The effect of prior removal of membrane sialic acid from E^s bearing C3b,Bb,P sites on the inhibitory activity of the E^{hu} membrane protein was examined with E^s from which 71% of sialic acid had been enzymatically released. Replicate samples of 1 × 10^7 E^sC3b,Bb,P and desialated E^sC3b,Bb,P were incubated for 20 min at 30°C in GVB with 20 mM EDTA alone or containing increasing concentrations of the inhibitory membrane protein, ranging from 0.88 to 14 ng/ml, and residual C3b,Bb,P sites were developed hemolytically as in the experiment depicted in Fig. 2. In a parallel experiment, additional replicate samples of normal and desialated E^s bearing C3b,Bb,P sites were incubated for 20 min at 30°C with increasing concentrations of β1H, ranging from 10 to 160 μg/ml. Inhibition by the membrane protein of 50% of the C3b,Bb,P sites on both normal and desialated E^s occurred with 4.2 ng/ml (Fig. 3 *left*). In contrast, the 50% inhibitory dose of β1H increased from 32 ng/ml for C3b,Bb,P sites on normal E^s to 104 ng/ml for sites on desialated E^s, indicating a reduction to one-third of its decay-dissociating activity (Fig. 3 *right*).

Because the inhibitory protein of the E^{hu} membrane accel-

Table 1. Residual ^{125}I-labeled Bb molecules per cell after treatment of E^sAC4b,3b,Bb,P with the inhibitory protein of the E^{hu} membrane

E^{hu} membrane protein,* ng	Bb molecules/ E^sAC4b,3b,Bb,P
None (unincubated)	1320
0	594
1.6	346
4.7	116
14.0	25

* Incubated for 20 min at 30°C.

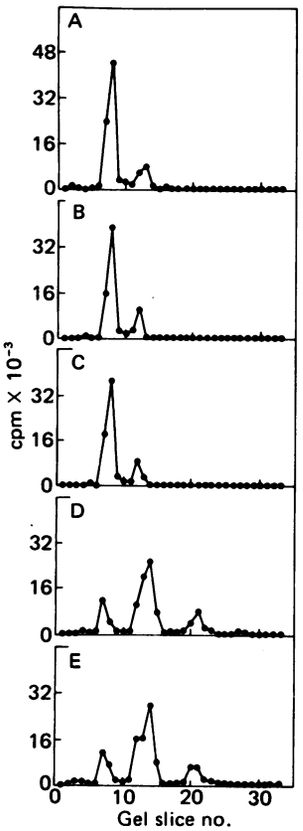

FIG. 4. Cleavage of the α polypeptide chain of ^{125}I-labeled C3b by C3bINA in the presence of the inhibitory protein of the E^{hu} membrane, as assessed by NaDodSO$_4$/polyacrylamide gel electrophoresis. ^{125}I-Labeled C3b was incubated with buffer alone (*A*), with C3bINA (*B*), with the inhibitory membrane protein (*C*), with C3bINA and the inhibitory membrane protein (*D*), and with C3bINA and β1H (*E*).

erated decay of C3b,Bb,P sites on E^s, its capacity to dissociate radiolabeled Bb from these sites was determined. E^sC3b (2.2×10^8) were incubated with 4 μg of P, 0.2 μg of $\overline{D}$, and 2 μg of ^{125}I-labeled B in 4.2 ml of DGVB^{++} for 45 min at 30°C, washed three times with ice-cold DGVB^{++}, and resuspended in 2.2 ml of DGVB^{++}. Replicate samples containing 5×10^7 cells were assessed at time zero for bound ^{125}I-labeled Bb and then incubated for 20 min at 30°C in 0.5 ml of DGVB^{++} alone or containing 14, 4.7, and 1.6 ng of the inhibitory membrane protein. The cells were washed three times with ice-cold DGVB^{++}, resuspended in 0.5 ml of DGVB^{++}, and assessed for residual cell-bound ^{125}I-labeled Bb. Specifically bound ^{125}I-labeled Bb was calculated after subtraction of the 32 molecules of ^{125}I-labeled B that bound per cell in the presence of 20 mM EDTA. ^{125}I-Labeled Bb dissociated from C3b,Bb,P sites on E^s during incubation in buffer alone, and the presence of the inhibitory membrane protein induced release of additional ^{125}I-labeled Bb in a dose-related manner (Table 1).

Inhibitory Protein of the E^{hu} Membrane as Cofactor for Cleavage of C3b by C3bINA. The capacity of the inhibitory protein of the E^{hu} membrane to serve as a cofactor for C3bINA-induced cleavage of the α polypeptide chain of C3b by C3bINA was examined. ^{125}I-Labeled C3b (50 μg/ml) in 0.04 ml of VBS was incubated for 30 min at 37°C alone, with 25 μg of C3bINA per ml, with 1 μg of membrane protein per ml, with C3bINA and the membrane protein, and with C3bINA and 1 μg of β1H per ml. After reduction with 0.2 M 2-mercaptoethanol, the samples of ^{125}I-labeled C3b were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis on 7% gels for analysis of the 110,000 M_r and 80,000 M_r α and β polypeptide chains. Neither C3bINA nor the inhibitory protein of the E^{hu} membrane alone induced cleavage of the α chain of ^{125}I-labeled C3b (Fig. 4). Treatment of ^{125}I-labeled C3b with both proteins resulted in a decrease of the 110,000 M_r α chain and the appearance of new radiolabeled peaks with apparent M_r of 65,000 and 40,000, which corresponded to the fragments of the α chain of ^{125}I-labeled C3b produced by C3bINA in the presence of β1H.

DISCUSSION

Discrimination between cells by the alternative complement pathway occurs after initial deposition of C3b and is related to modulation by surface constituents of the capacity of C3b to function as a subunit of C3b,Bb. Two biochemical entities that are present on cell membranes, sialic acid (9, 11, 16, 17) and sulfated mucopolysaccharides (18), regulate the function of bound C3b indirectly through promotion of its interaction with the plasma control proteins, β1H and C3bINA. The isolation of a protein from the E^{hu} membrane that can interact directly with C3b to disassemble C3b,Bb and to augment cleavage-inactivation of C3b by C3bINA suggests that some homologous membranes may have intrinsic means for preventing their recognition by the alternative pathway.

The inability of E^{hu} that had been 80% desialated to induce activation of the alternative complement pathway, as assessed by lysis in normal human serum, prompted a search for additional membrane-associated control mechanisms. Elution of E^{hu} membranes with low- and high-ionic-strength buffers, conditions that release approximately 24% and 4%, respectively, of associated proteins (28), did not release inhibitory activity for C3b,Bb,P; solubilization of membrane protein with the use of NP-40 did release activity, suggesting that the inhibitory protein is intrinsic to the E^{hu} membrane. The inhibitory protein was adsorbed by Sepharose-lentil lectin and Sepharose-C3, indicating that it is a glycoprotein with an affinity for C3. Although the inhibitory protein of the E^{hu} membrane filtered in the presence of NP-40 with an apparent M_r of $1\text{–}1.2 \times 10^6$, the final purified material presented a single stained band on NaDodSO$_4$/polyacrylamide gel electrophoresis with an apparent M_r of 205,000 with or without prior reduction of disulfide bonds (Fig. 1). Thus, the protein may normally exist as an oligomer composed of four to six noncovalently linked polypeptides of 205,000 M_r, although its size characteristics on gel filtration might not be indicative of its state when residing in the E^{hu} membrane.

Because the action of the inhibitory protein of the E^{hu} membrane was directed at the functional integrity of the C3b,Bb,P, the time course of this effect was assessed at different concentrations of the protein (Fig. 2). Accelerated loss of functional C3b,Bb,P sites on E^s mediated by the membrane protein was dose-related and first order, indicating an immediate and continuous action on these sites. The capacity of the inhibitory membrane protein to release ^{125}I-labeled Bb from C3b,Bb,P sites (Table 1) demonstrated that the mechanism of inhibition is decay-dissociation of the C3b,Bb complex, probably reflecting a capacity to bind to C3b. The additional function of serving as a cofactor for cleavage of the α polypeptide chain of C3b by C3bINA (Fig. 4) indicates that the inhibitory membrane protein also can prevent the generation of C3b,Bb sites by augmenting inactivation of C3b.

Although the inhibitory protein of the E^{hu} membrane and β1H are similar in their decay-dissociating and C3bINA cofactor functions, they differed in their specific activities and relative dependence on membrane sialic acid for function. Whereas 4.2 ng of the membrane protein per ml decay-dissociated 50% of C3b,Bb,P sites on E^s in 20 min, almost 8 times more β1H, 32 ng/ml, was required to effect this extent of inhibition (Fig. 3). Moreover, prior removal of 71% of membrane sialic acid from E^s bearing C3b,Bb,P sites did not impair the inhibitory action of the E^{hu} membrane protein but increased

the amount of β1H required for 50% decay-dissociation to 104 ng/ml (Fig. 3). Thus, the inhibitory protein of the E^{hu} membrane differs from the plasma protein, β1H, by its greater M_r, higher specific activity, and independence of decay-dissociating activity from membrane sialic acid. The inhibitory membrane protein may be related to an activity obtained by butanol extraction of E membranes from several species that is capable of accelerating decay of the classical pathway C3 convertase (29).

The human alternative complement pathway can serve as a mechanism in the nonimmune host for recognition of cell surfaces presented by a variety of microorganisms and by some heterologous mammalian cells. Because recognition of cells by this system may eventuate in phagocytosis or cytolysis, autologous cells must have the capacity to regulate strictly the formation and function of the C3b,Bb on their membranes. The presence in the E^{hu} membrane of a protein that can impair the function of membrane-bound C3b and C3b,Bb could represent one molecular basis for preventing inappropriate self-recognition.

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